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(54) Title: PURIFIED HCV AND HCV PROTEINS AND PEPTIDES

(57) Abstract

Intact hepatitis C virus (HCV) particles, purified HCV proteins, and glycopeptide antigens specific to HCV particles are disclosed. The virus particles, in inactivated or attenuated form, are useful in a vaccine. The purified proteins, and glycopeptide antigens are useful in a diagnostic system, for detection of human HCV antisera, and in vaccine compositions. Also disclosed are antibodies specific against the glycopeptide antigens.

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PURIFIED HCV AND HCV PROTEINS AND PEPTIDES

1. Field of the Invention

The present invention relates to purified hepatitis C virus (HCV), mature virus proteins and glycopeptide antigens isolated from the virus, and vaccine and diagnostic compositions which utilize the particles, proteins and antigens.

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3. Background of the Invention

Non-A, Non-B hepatitis has long been recognized as a virus-induced disease, distinct from other forms of viral-associated liver diseases, including hepatitis A virus

(HAV) and B virus (HBV), and hepatitis-induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). NANBH virus is implicated in greater than 90% of all post-transfusion hepatitis cases, and is responsible for the induction of chronic hepatitis in 40-50% of infected individuals. There is growing evidence for at least two distinct types of NANBH viruses. One is an enterically transmitted virus, and may be seen in epidemic form where sanitation conditions are poor. The other is parenterally transmitted NANBH virus, now commonly referred to as hepatitis C virus (HCV), which is a major source of hepatitis in transfused blood.

The limited availability of an animal model (HCV infects only chimpanzees and humans) and the absence of an in vitro tissue culture model suitable for growing HCV has limited the isolation and characterization of mature HCV virus. Such particles would be useful for production of inactivated or attenuated HCV for vaccine and diagnostic purposes, and for isolation and identification of intact, mature HCV proteins, and glycosylated HCV antigens.

4. Summary of the Invention

It is therefore a general object of the invention to provide isolated, intact HCV virus particles, and protein and glycopeptide antigens obtainable therefrom.

The invention includes, in one aspect, purified HCV virus particles characterized by: (a) a single-stranded RNA genome; (b) a flavivirus type structure having virus particle sizes between about 30-60 nm, enveloped capsid structures, external stalks 2-5 nm in length and width, and an icosahedron symmetry, and (c) immunospecific reaction with HCV-infected individuals. In on mbodiment, the RNA

genome of the particles contains a region with the sequence shown in Figure 4.

The particles are useful, in inactivated form, in a vaccine composition, or for generating monoclonal antibodies specific against HCV virus antigens, and in particular, against glycopeptide antigens.

The protein components of the virus particles, when fractionated and purified, yield mature HCV proteins, such as purified, mature C, e1 (gp35), e2 (gp70), and the HCV counterparts to NS2, NS3, NS4, or NS5, which may be substantially free of HCV genomic RNA, and non-viral serum and cell proteins normally associated with HCV virus infection in humans.

A purified protein or protein mixture is useful in a vaccine composition, for generating monoclonal antibodies specific against HCV viral proteins, and in a diagnostic system for detection of human anti-HCV anti-sera.

The virus particles or proteins isolated therefrom also provide a source of HCV-specific glycopeptide antigens, i.e., glycopeptide regions of a glycosylated HCV proteins, such as gp35 and gp70. The antigens are useful in a diagnostic system for detecting human sera from HCV-infected individuals.

In another aspect, the invention includes an antibody specific against the HCV glycopeptide antigen. The antibody is useful as a diagnostic reagent, for detecting the presence of HCV antigens in HCV-infected human sera.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figures 1A and 1B are electron photomicrographs of HCV particles at two different magnifications, where the bars in the figures indicate 50 nm;

Figures 2A-2E are electron photomicrographs of HCV particles, where Figures 2A-2D (bar = 23 nm) show typical variation in size and shape, with a core-like structure being evident in Figure 2C, and Figure 2E (bar = 17 nm) is taken at higher magnification and shows external surface stalks on the virus particle;

Figures 3A-3C are electron photomicrographs of HCV particles, showing bar-like structures within the virus core (3A and 3C) and prominent envelope structures with external stalk projections (3B);

Figure 4 gives the sequence of a cDNA region of an HCV strain isolated from HCV-infected liver, also showing nucleotide divergence with earlier published HCV sequences, designated JT and PT;

Figure 5 shows electrophoretic patterns of PCR products of RNA from various immortalized CU chimpanzee hepatocyte cell lines derived from HCV-infected chimpanzee hepatocytes (lanes 1-8), and from chimpanzee liver RNA during the acute phase of HCV infection (lane 9); and

Figure 6 shows electrophoretic patterns of PCR products of RNA from various HCV-infected CHMP cells (lanes 1-12 and 14), from the inoculum used to infect the cells (lane 13), from chimpanzee liver RNA during the acute phase of HCV infection (lanes 15 and 18), and from an HCV cloned fragment.

Detailed Description of the Invention

I. HCV Virus Particles

This section describes cell culture sources of HCV particles having the characteristics:

(a) a single-strand RNA genome;

(b) a flavivirus type structure having virus particle sizes between about 30-60 nm, enveloped capsid structures, external stalks 2-5 nm in length and width, and an icosahedron symmetry, and

(c) immunospecific reaction with HCV-infected individuals.

A. Cell culture sources.

One cell-culture source of HCV virus particles is a cultured primary hepatocytes derived from the liver of an HCV-infected chimpanzee or human, and cultured under conditions which maintain the differentiated state of the infected cells for 3-4 weeks. Methods for preparing primary primate hepatocytes for culture, and culture medium conditions effective to preserve liver-specific functions for extended periods in culture have been described by the inventors (Lanford, 1989)

Details of the primary cell culture methods are given in Example 1. Briefly, liver tissue obtained from an HCV-infected chimpanzee or human is perfused and hepatocytes are dislodged by treatment with collagenase. The cells are washed several times, then plated on culture plates at a density of about 5 x 105 to 5 x 10^6 cells per 60 mm plate. The hepatocytes are maintained in serum-free medium (SFM) which has been specifically designed to allow the cells to grow in culture in a highly differentiated state, as

evidenced by the continued production and secretion in culture of liver-specific proteins.

One preferred SFM is composed of Williams' medium E (WME) supplemented with 10 mM HEPES, pH 7.4, 50 ug gentamicin, and the following supplements: EGF (epidermal growth factor), insulin, glucagon, BSA (bovine serum albumin), soybean lipids, linoleic acid, hydrocortisone, selenium, cholera toxin, LGF (liver growth factor, a glysyl-histidyl-lysine tripeptide), ECGS (endothelial cell growth supplement), transferrin, ethanolamine, prolactin, somatotropin, and TRF (thyrotropin-releasing factor), in the proportions given in Example 1. The sources of these materials are given elsewhere (Lanford). The cells are maintained in the SFM under standard cell culture conditions. The medium is changed, e.g., 24 hours after isolation and every 48 hours thereafter, during the culture period. Under these conditions, the cells undergo 2-4 rounds of replication in the first several days of culture, e.g., within 7-10 days, and thereafter continue to function as liver-specific cells in culture, but without appreciable signs of cell replication, for 3-4 weeks total culture period. Thereafter, the virus-infected cells gradually lose hepatocyte differentiation, as evidenced by a decline in the production of liver-specific proteins.

The differentiation of the primary hepatocytes in culture can be assessed by following changes in the production and secretion of liver-specific proteins. In one approach described in Example 1, proteins from the culture medium are fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fractionated proteins are detected by immunoblotting, using antibodies directed against the proteins of inter st. In

a second approach, also described in Example 1, radio-labeled cell culture proteins are immunoprecipitated with immobilized, protein-specific antibodies, and the precipitated antibodies are then fractionated by SDS-PAGE. In both cases, analysis of the gel patterns showed that the hepatocytes produced a number of liver-specific proteins in approximate relative proportions to that found in plasma for up to 3-4 weeks, then showed a gradual decline in the amount of protein produced. The decline in liver-specific protein production, such as apolipoprotein production, paralleled a degeneration in the hepatocyte cultures.

HCV particles were obtained from the HCV-infected hepatocytes in culture for up to three-seven weeks after the infected cells were placed in culture (Table 1).

Another source of HCV particles, in accordance with the invention, is chimpanzee or human primary hepatocytes which are infected in vitro with HCV inoculum. A method of obtaining and culturing hepatocytes from uninfected chimpanzees is given in Example 5, and generally follows the culture method used to form stable, differentiated primary hepatocytes derived from HCV-infected liver cells. The cells are infected with a pooled inoculum of plasma samples from several chimpanzees with known acute HCV infection, as described in Example 5.

Table 1 below compares viral counts obtained from cultured hepatocytes which were derived either from HCV-infected chimpanzees (PTTx196, PTTx174, PTTx268, and PTTx198) or from non-infected chimpanzees, whose cultured hepatocytes were infected in vitro (PTTx266 and PTTx344), where culture samples were taken at the culture times indicated. The results show the ability to replicate, isolate and purify the HCV virus in hepatocytes derived

from both HCV-infected and non-infected HCV-infectable animals.

Table 1

ANIMAL	DAY OF CULTURE	PARTICLES X10^6/ml
PTT x196 Russel	1	1.2
	3	3.2
	5	0.6
	7	3.6
	11	1.6
PTT x174 Orville	11	0.6
	13	1.0
	15	1.0
	17	6.0
PTT x268 Co Co	1	0
	3	0
	5	0
	7	2.1
	12	11.4
	14	15.7
	16	7.1
	20	11.6
	22	2.1
	24	3.6
PTT x266 Joshua	1	0
	2	0
	3	0
	6	2.0
	9	0
	12	5.6
	15	0
	18	2.4
	21	2.0
PTT x344 Melissa	1	ND
	3	ND
	5	0
	7	0
	9	0
	12	6.3

ANIMAL	DAY OF CULTURE	PARTICLES $X10^6$/ml
PTT x198 Ronald	1	1.6
	3	1.2
	5	0.3
	7	0
	9	0
	11	0
	13	0.3
	15	0.4
	18	6.0
	20	5.2
	22	5.6
	25	0
	27	7.6
	29	13.2
	32	8.6
	34	13.2
	36	9.2
	39	4.4
	41	2.4
	44	6.8
	47	0.4
	49	0
	51	4.4
	54	0

A third source of HCV particles, also in accordance with the invention, is immortalized chimpanzee or human hepatocytes which are infected in vitro with HCV after immortalization. Immortalization is achieved by introducing an oncogene into stable, non-infected or HCV-infected primate hepatocytes in culture, as detailed in the companion patent application for "An Immortalized Hepatocyte Cell Line", and illustrated in Example 6. Briefly, hepatocytes obtained from non-infected or HCV-infected chimpanzees (or humans) are cultured, as above, under conditions which allow expression a liver-specific proteins for extended culture periods. During the first 2-4 rounds of replication of the cultured cells, the culture is exposed to a

virus or plasmid vector containing a suitable oncogene, such as the SV40 large T antigen oncogene, and immortalized cells are selected on the basis of continued growth beyond initial 2-4 rounds of replication, when non-immortalized cells are essentially non-replicative.

To select an immortalized cell line capable of supporting HCV infection and replication, several immortalized cell lines from above are infected with an HCV inoculum, and the individual cell lines are assayed for the presence of HCV RNA, using PCR methods such as detailed in Example 6.

Figure 5 shows a gel analysis of the PCR products from HCV-infected CU cell lines (produced by immortalization of hepatocytes from an HCV-infected chimpanzee). Lanes 1-8 are CU1, CU3, CU4, CU5, CU6, CU8, CU9 and CU12, respectively. Lane 9 is a positive control of chimpanzee x198 liver RNA during the acute phase of HCV infection and was processed identically as the CU RNA samples. Lanes 10 and 11 are the cDNA and PCR negative controls to demonstrate the lack of contamination during the PCR assay. Lane 12 is lambda DNA cleaved with HindIII as size markers. Lane 5 (CU6) and 9 (PCR positive control) show a positive reaction. All lanes have a lower band that represents the primers used in the PCR reaction. Positive reactions were obtained with CU6 cell line, the inoculum used to infect the cell lines, and each of the positive controls. The negative controlled were negative indicating that no contamination occurred during the PCR reaction.

Figure 6 shows a gel analysis of PCR products from HCV-infected CHMP cell lines (produced by immortalization of hepatocytes derived from n n-infected chimpanzees hepatocytes). Lanes 1-12 represent CHMP 1.21, 1.22, 1.23,

1.24, 1.25,1.26, 1.27, 1.28, 1.29, 1.30, 1.31 and 1.32, respectively. Lane 13 is the PCR analysis of the inoculum used to infect both the CU and CHMP cell lines. Lane 14 is CHMP 2.02. Lane 15, 18 and 19 are PCR positive controls. Lane 15 and 18 are PTTx198 liver RNA as described for Figure 6. Lanes 16 and I7 are cDNA and PCR negative controls, respectively. Lane 19 is a PCR positive control consisting of a gel purified band from a cloned fragment of HCV homologous to the PCR primers used in this assay. Lane 20 is HindIII digested lambda DNA as size markers.

Positive reactions were obtained with CHMP 1.27 and CHMP 2.02 cell lines, the inoculum used to infect the cell lines, and each of the positive controls. The negative controls were negative indicating that no contamination occurred during the PCR reaction.

Thus, of the 20 CU and CHMP cell lines tested, three were permissive for infection with and replication of HCV. The cell lines are CHMP 1.27, CHMP 2.02 and CU6. These results demonstrate that immortalized chimpanzee hepatocytes, whether derived from non-infected or HCV-infected animals, are infectable with HCV, and support replication of HCV, for use in the production of HCV.

B. Isolation of Virus Particles

Virus particles can be isolated from HCV infected chimpanzee or human hepatocytes in culture by gradient centrifugation methods, as described in Example 2. In one preferred method, culture medium is clarified by low-speed centrifugation, then separated from soluble culture-medium components by centrifugation through a 20% sucrose layer by high-speed centrifugation. The material is further

purified by centrifugation onto a 68% sucrose cushion at high speed.

Other methods for separating virus particles from soluble culture-medium components may be used. For example, clarified culture medium can be passed through a size-exclusion matrix, to separate soluble components by size exclusion.

Alternatively, the clarified culture medium can be passed through an ultrafiltration membrane having a 10-20 nm pore size capable of retaining virus particles, but passing solute (non-particulate) culture medium components.

C. Virus Particle Characteristics

Purified HCV virus particles from above were examined for morphological features, as detailed in Example 2. Figures 1A and 1B are electron photomicrographs of HCV particles from cells derived from PTTx174 chimpanzee, at two different magnifications, where the bars in the figures indicate 50 nm. Figures 2A-2E are electron photomicrographs showing further structural features and variations in HCV particles (composite of HCV's from liver derived from different HCV-infected chimpanzees), where the bars in Figures 2A-2D represent 23 nm. A core-like structure is evident in Figure 2C. Figure 2E (bar = 17 nm) is taken at higher magnification and shows external surface stalks on the virus particle;

The electron micrographs of HCV particles (from hepatocytes derived from the liver of PTTx266 chimpanzee) in Figures 3A-3C show bar-like structures within the virus core (3A and 3C) and prominent envelope structures with external stalk projections (3B).

Summarizing the structural features, the HCV particles have:

(a) an approximate average outside diameter of 39-46 nm, but with a wide range in particle size (30-60nm);

(b) an internal core structure approximately 35-40nm in diameter;

(c) a dense intra-core bar-like structure within some of the particles;

(d) external "stalks" and "knobs" protruding from the envelope, which measure approximately 2 to 5 nm in length, and 2 to 5 nm in width, respectively;

(e) particle envelopes; and

(f) icosahedron symmetry.

The virus particles isolated and visualized electron microscopically from the tissue cultured hepatocyte medium displayed a morphology similar to the genus flaviviruses of the Togaviridae family. Togavirus virions consist of a lipid-containing envelope with surface projections surrounding a spherical nucleocapsid with proven or presumed icosahedral symmetry. Virions are 40 to 70 nm in diameter. The genome consists of one molecule of positive-sense infectious ssRNA of MW $4x10^6$. The viruses exhibit pH-dependent hemagglutinating activity. Replication takes place in the cytoplasm, and assembly involves proven or presumed budding through host cell membranes. (See, for example, Murphy or Schlesinger).

Similarly, the chloroform sensitive nature of the HCV virus, indicative of a lipid-containing envelope (Feinstone), as well as the apparent size distribution of the HCV agent (30-60nm) determined by selective filtration techniques (He), are features compatible with our ultra-

structural observations of an enveloped virus, whose size range is observed to be 39-60nm.

D. Infective HCV Particles

The HCV particles isolated as disclosed above are also infectious. This is evidenced by the ability of cell culture medium from the HCV-infected primary or immortalized hepatocytes to produce HCV infection in chimpanzees. Details of one study in which culture medium from HCV-infected primary hepatocytes is used to infect chimpanzees are given in Example 4. Weekly blood samples and periodic liver biopsies showed active hepatitis infection at 16-20 weeks after initial infection.

D. Virus Particle Genome

The virus particles described in Example 2 were isolated from primary hepatocytes derived from the liver of a chimpanzee infected with the Hutchinson strain of HCV. In order to detect and sequence the RNA genome of the infective HCV, total RNA isolated from the biopsied liver sample was amplified by polymerase chain reaction (PCR) methods, using HCV-specific primers, and the amplified fragment was cloned and sequenced, according to methods detailed in the Example 4.

The amplified, cloned HCV sequence (termed BTR 623) includes 623 nucleotides of HCV specific sequence. This isolate was compared to sequences of previously published JI and PT HCV clones (Kubo), as shown in Figure 4. The sequences given in this figure indicate that the HCV strain used in these studies has significant sequence divergence with the published isolates. The greatest level of diverg nce was seen with the J1 sequence. BTR 623 had

96.2% nucleotide and 97.4% amino acid homology with PT, and 79.8% nucleotide and 93.8% amino acid homology with J1.

II. HCV Antigens

A. HCV proteins

Heretofore, HCV virus proteins have been obtained only in recombinant form, using expression vectors with known HCV coding sequences to express HCV proteins or peptides in a suitable expression system. Such recombinant proteins are likely to differ from mature, intact virus proteins in glycosylation, acetylation, and phosphorylation modifications, as well as terminal residue modifications or cleavages. These modifications, particularly glycosylation features, are likely to be important in virus interactions with host cells (Schlesinger), and in the host's immune response to the virus.

The present invention allows glycosylation and other post-translation modifications in intact HCV virus proteins to be identified and isolated. The glycosylation sites can be identified by standard Western blotting procedures (Harlow), in which isolated HCV virus is fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the virus protein bands are probed with HCV antisera, to identify immunoreactive viral proteins. Similarly, the viral proteins can be cleaved by selected proteases, prior to Western blotting, to identify immunoreactive peptide fragments. The immunoreactive proteins or protein fragments can be identified by amino acid sequencing of the Western blot bands, either directly, or after additional band purification, if necessary.

From the identification of the proteins, and from known consensus sequences for glycosylation sites, e.g.,

Asp-X-Ser/Thr, proteins or fragments thereof which contain glycosylated residues can be identified. Alternatively, such proteins or fragments can be identified by comparing 2-dimensional gel electrophoresis patterns of virus proteins or peptide fragments before and after treatment with selected glycosidases. Two HCV proteins, gp35, and gp70, have been identified by others from coupled *in vitro* translation end studies, as containing glycosylation sites based on their sensitivity to endoHglycosidase.

After identifying glycosylated proteins or peptides of interest, the isolated viral particles are used as a source of the selected glycoprotein or peptide. Protein or peptide isolation from the viral particles can be carried out by standard methods, such as ion exchange and size-exclusion chromatography, and HPLC purification. The present invention contemplates in particular, mature gp35 (E1) and gp70 (E2) HCV proteins with native glycosylation, and mature glycosylated peptides from these two proteins. The proteins and peptides are useful in a diagnostic system and in a vaccine composition, as described in Section IV below.

III. Anti-HCV Antibodies

In another aspect, the invention includes polyclonal or monoclonal antibodies specific against mature HCV particles and protein components thereof. The antibodies are defined by specific immunoreactivity with features of HCV particles, or proteins or peptide fragments thereof, due to normal post-translational modification. That is, the antibodies are immunoreactive only with recombinant HCV proteins which contain normal virus post-translational modifications.

Polyclonal antibodies can be prepared, in accordance with one embodiment, by affinity chromotography, using the glycopeptide antigens identified from above immobilized on a solid support, for extracting immunoreactive antibodies in naturally-infected human or chimpanzee HCV anti-sera or antisera generated specifically against the glycopeptide antigen.

Alternatively, the glycosylated proteins or peptides from above can be used to produce monoclonal antibodies, employing standard methods (Harlow). Briefly, the protein or peptide antigen is used to elicit an immune response in an animal, such as a mouse or rabbit, B lymphocytes from the spleen of the immunized animal are immortalized with a suitable hybridoma partner, and selection of desired hybridomas is made on the basis of immunoreactivity with the glycoprotein or peptide of interest. The antibodies made by the selected hybridoma are useful in a diagnostic method, for screening human sera for HCV infection, and in a vaccine composition, for producing active immunity, as discussed in Section 4.

IV. Utility

A. Detection of HCV Antisera

The virus particles, and proteins and glycosylated peptides derived therefrom are useful as diagnostic reagents for detecting anti-HCV antibodies present in HCV-infected sera. As noted above, the mature particles, proteins and glycosylated peptides offer the advantage over recombinantly prepared HCV peptides and proteins in that in addition to peptide antigens, the agents provide potentially unique antigenic sites associated with mature viral proteins, such as glycosylated peptides.

In one preferred diagnostic configuration, test serum is reacted with a solid phase reagent having surface-bound viral proteins or peptides. After binding anti-HCV antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-HCV antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric or colorimetric substrate.

The solid surface reagent in the above assay prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group.

In a second diagnostic configuration, known as a homogeneous assay, antibody binding to a solid support produces some change in the reaction medium which can be directly detected in the medium. Known general types of homogeneous assays proposed heretofore include (a) spin-labeled reporters, where antibody binding to the antigen is detected by a change in reported mobility (broadening of the spin splitting peaks), (b) fluorescent reporters, where binding is detected by a change in fluorescence efficiency, (c) enzyme reporters, where antibody binding effects

enzyme/substrate interactions, and (d) liposome-bound reporters, where binding leads to liposome lysis and release of encapsulated reporter. The adaptation of these methods to the protein antigen of the present invention follows conventional methods for preparing homogeneous assay reagents.

In each of the assays described above, the assay method involves reacting the serum from a test individual with the protein antigen and examining the antigen for the presence of bound antibody. The examining may involve attaching a labeled anti-human antibody to the antibody being examined, either IgM (acute phase) or IgG (convalescent or chronic phase), and measuring the amount of reporter bound to the solid support, as in the first method, or may involve observing the effect of antibody binding on a homogeneous assay reagent, as in the second method.

Also forming part of the invention is an assay system or kit for carrying out the assay method just described. The kit generally includes a support with surface-bound mature virus particle, protein or peptide, and a reporter-labeled anti-human antibody for detecting surface-bound anti-409-1-1 antibody.

B. HCV Vaccine

The virus particles, or mature processed proteins or antigenic peptides therefrom can be formulated for use in a HCV vaccine. The vaccine can be formulated by standard methods, for example, in a suitable diluent such as water, saline, buffered salines, complete or incomplete adjuvants, and th like. The immunogen is administered using standard t chniqu s for antibody induction, such as by subcutaneous

administration of physiologically compatible, sterile solutions containing inactivated or attenuated virus particles or antigens. An immune response producing amount of virus particles is typically administered per vaccinizing injection, typically in a volume of one milliliter or less.

A specific example of a vaccine composition includes, in a pharmacologically acceptable adjuvant, intact virus particles. This vaccine contains a combination of core and envelope antigens. Another specific example includes, in a pharmaceutically acceptable adjuvant, a purified mature virus protein, such as the gp35 or gp70 protein, or a combination of C (core) protein with envelope protein, such as the gp35 or gp70 proteins.

Although the invention has been described with respect to particular methods, cell line, HCV strains, and applications, it will be apparent that various changes and modification can be made without departing from the invention.

Example 1

Primary HCV-Infected Chimpanzee Hepatocytes

A. Liver Samples

A parenteral HCV virus infection was induced in chimpanzee PTTx7, a 14-year old female, by inoculation with 5 ml of a 20-fold concentrate of acute phase plasma of unknown titer derived from a second chimpanzee passage of the Hutchinson strain of HCV (obtained from Dr. K Burk, Biotech Resources, Inc., San Antonio, TX)) was monitored by ALT/AST (alanine aminotransferase/aspartate aminotransferase) enzyme fluctuations from w ekly blood samples and by histopathologic examination of periodic liver needle punch

biopsies, according to published methods (Valenza). The "PTT" animal designations used herein identify individual chimpanzees housed at the Southwest Foundation for Biomedical Research, San Antonio, TX.

All biopsies were processed identically using conventional techniques. Immediately after harvesting, the liver biopsies were fixed for 1-3 hours in neutral buffered 3.7% formalin, processed manually according to standard procedures, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin. All sections were examined histologically by the same board certified veterinary pathologist.

Since the onset of clinical hepatitis was significantly delayed, a second inoculation of 1.5 ml ($10^{2.5}$ CID_{50}) of the original HCV virus Hutchinson inoculum was administered at week 10 to assure infection. The appearance of elevated ALT on week 12 indicated that the second inoculum either potentiated the primary infection or was not required. The ALT profile of the animal exhibited a rise above normal values from 12-19 weeks post inoculation, and a second ALT elevation occurred on week 39.

A liver punch biopsy taken after ALT elevations (week 19) revealed an increased number of lymphocytes in portal areas and in the parenchyma of the liver. Associated with the parenchymal lesions were necrotic hepatocytes. The hepatocytes around central vein areas were often lightly stained and granular with minimal swelling of the cytoplasm. All these changes described indicated minimal, lymphocytic, multifocal, viral hepatitis.

Liver wedge surgery was performed on week 14 at the onset of definitive ALT elevation. Ketamine hydrochloride was used as the immobilizing and prean sthetic agent.

Surgery was performed under general anesthesia with non-hepatotoxic sodium pentobarbital. A liver wedge of approximately 10 g was perfused using a modification of established protocols (Maslansky). Microscopic examination of liver tissue taken at this time revealed occasional collections of lymphocytes and macrophages in hepatic triads and in focal parenchymal areas. There were no other changes indicating a significant inflammatory response. Although minimal inflammation was present, this finding could be representative of normal liver tissue.

A two-step perfusion procedure was employed with all solutions maintained at 37°C throughout the perfusion procedure. The initial perfusion lasted 10 minutes using 1 liter of Ca++, Mg++ -free Hanks Balanced salt solution supplemented with 10 mM HEPES (pH 7.4), 0.5 mM EDTA, and 100 μg/ml gentamicin sulfate. The next perfusion was for 20 minutes at approximately 60 ml/min. of Williams Medium E (WME) supplemented with 10 mM HEPES (pH 7.4), 100 μg/ml gentamicin sulfate, and 200 units/ml collagenase Type I (300 units/mg, Sigma, St. Louis. MO).

The liver capsule from above was removed with fine forceps and hepatocytes were dislodged by gentle agitation in 100 ml of the above collagenase solution. The hepatocyte suspension was filtered through several layers of gauze pads into an equal volume of cold Williams Medium E (WME) containing 5% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), and 100 μg/ml gentamicin sulfate. Hepatocytes were sedimented at 50 x g for 5 minutes and cell pellets were resuspended in WME 5% FBS. Sedimentation was repeated twice, pellets were resuspended in 10 ml WME 5% FBS, and viability and cell density were determined by trypan blue exclusion.

B. Cell Culture Conditions

PRIMARIA plates (Falcon, Becton-Dickinson, Lincoln Park, NJ) were coated with rat tail collagen (Michalopoulos) for 6 minutes at room temperature, the excess collagen was removed, and plates were dried overnight under U.V. light. Viable cells were plated at a density of 3-4 x 10^6 cells/60mm dish. Cell attachment occurred during a 3-hour incubation at 37°C, 10% CO_2 in WME, 5% FBS, at which time cell monolayers were gently washed one time with WME and re-fed with the serum-free medium formulation described below. The medium was changed 24 hours after isolation and at 48 hour intervals thereafter.

The cultured hepatocytes displayed a typical hepatocyte morphology as observed by phase-contrast microscopy on day 5 of culture. This morphology was maintained until days 21-28 when the cultures exhibited a degenerative process.

In this and the other examples below, the serum-free media (SFM) formulation utilized a basal medium supplemented with 10 mM HEPES, pH 7.4, 2.75 mg/ml $NaHCO_3$, and 50 μg/ml gentamicin, together with the supplements as listed below. In the described media of Table 2, Williams Medium E (WME) served as a basal medium. Although WME is presently preferred as the basal medium of the serum-free medium other commercial media formulations can be expected to give satisfactory results. For instance, a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Salas-Prato) or RPMI 1640 (Gibco) (Enat, Sell should give satisfactory results when supplemented with the supplements listed in Table 2.

Table 2

Supplement	Medium Concentration
EGF	100 ng/ml
Insulin	10 μg/ml
Glucagon	4 μg/ml
BSA	0.5 mg/ml
Linoleic Acid	5 μg/ml
Hydrocortisone	10^{-6} M
Selenium	10^{-8} M
Cholera Toxin	2 ng/ml
LGF	20 ng/ml
Transferrin	5 μg/ml
Ethanolamine	10^{-6} M
Prolactin	100 ng/ml
Somatotropin	1 μg/ml
TRF	10^{-6} M

To prepare the media, the supplements were added in the following quantities in Table 2 to 500 ml of WME in a sterile plastic bottle:

5 ml	50 mg/ml BSA (bovine serum albumin), 500 μg/ml Linoleic Acid
0.5 ml	5 mg/ml Insulin
0.5 ml	5 mg/ml Insulin, 5 mg/ml Transferrin, and 5 μg/ml Selenium (ITS)
50 μl	10^{-2} M Hydrocortisone
5 μl	200 μg/ml Cholera toxin
0.5 ml	100 μg/ml EGF (epidermal growth factor)
50 μl	10^{-2} M Ethanolamine
0.5 ml	1 mg/ml Somatotropin

50 µl 1 mg/ml Prolactin

0.5 ml 10^{-3} M Thyrotropin Releasing Hormone

50 µl 200 µg/ml LGF (liver growth factor, i.e., glycyl-histidyl-lysine)

1 ml 2.0 mg/ml Glucagon

WME was purchased with L-glutamine and without $NaHCO_3$ from Hazelton Research Products, Inc. (Denver, Pennsylvania). Supplements, including growth factors and hormones were obtained from Sigma (St. Louis, MO) or Collaborative Research (Bedford, MA).

C. Secretory Protein Production

The synthesis and secretion of albumin, apolipoprotein A-I and apolipoprotein E were monitored by immunoblotting of sequential aliquots of tissue culture medium, according to standard methods (Haslow). Briefly, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were electrophoretically transferred to Nylon-X nitrocellulose filters (Fisher) at 100 mA current for 16 hours at 4°C. Unoccupied binding sites were blocked in 10% nonfat dry milk in phosphate buffered saline (PBS) for 2 hours at 37° C in PBS-milk-Tween (PBS containing 5% nonfat dry milk, 0.3% Tween-20), using primary antibodies directed against each of the specific proteins. Membranes were washed three times with PBS-Tween and incubated 1 hour at 37°C in PBS-milk-Tween with [^{125}I]-protein A (8.5 µCi/µg, New England Nuclear, Boston, MA). Membranes were washed three times with PBS-Tween and air dried. Immunoblots were autoradiographed at -85° C on XAR-5 film (Kodak, Rochester, N.Y.) with intensifying screens.

The levels of apolipoproteins A-I and E increased in the cultures up to day 13, remained constant from day 13-28, and declined from day 28-45. Albumin detected by this

immunoblot procedure remained at constant levels throughout the culture period. Although albumin is a marker for differentiated hepatocytes, it is not as stringent of a marker for the differentiated state as is lipoprotein synthesis.

The decline in lipoprotein synthesis after 28 days in culture paralleled a degeneration in the hepatocyte cultures. The degeneration of primary hepatocytes after 3-4 weeks of culture was evident in cultures derived form two different HCV-infected chimpanzees. Normal hepatocyte cultures generally survive more than 100 days in the serum free media. It thus appears that the degenerative process of HCV-infected primary hepatocytes may be due to viral induced cytopathic effect.

To further characterize the differentiated state of the hepatocytes in vitro, the de novo synthesis of liver specific plasma proteins was analyzed. On day 17, cultures were labeled for 24 hours with [^{35}S] methionine (>800 μCi/mmol, ICN) for 24 hours. Medium was filtered and mixed with 1/10 volume of 10x CHAPS extraction buffer [final concentration 1.0% CHAPS (CalBiochem), 0.25mM phenylmethyl sulfonyl fluoride, 10mM EDTA, 0.05 M Tris (pH 8.0), 0.1 M NaCl, 100 μM leupeptin] and incubated for 1 hour at 4° C with agitation. Commercially obtained antibodies (CalBiochem, San Diego, CA and Boehringer Mannheim, Indianapolis, IN) directed against human plasma proteins (20μl) were bound to protein A-agarose beads (50 μl, Repligen) for 1 hour in CHAPS extraction buffer on ice. The beads were washed two times with detergent wash buffer [CHAPS extraction buffer plus 1% deoxycholic acid and 0.1% SDS] and were incubated with the labeled medium overnight at 4°C with agitation.

The beads were pelleted and washed three times with detergent wash buffer.

Bound proteins from the culture medium were eluted with 50 µl electrophoresis sample buffer containing 2% SDS and 2% 2-mercaptoethanol, heated at 100°C for 10 min. and analyzed by SDS-PAGE. Gels were processed for fluorography with Autofluor (National Diagnostics, Somerville, NJ), dried, and autoradiographed at -85° C on XAR-5 film.

Analysis of the gel patterns indicates that the amount of plasma proteins synthesized in vitro reflects the concentrations found in plasma. The intensities of the polypeptide bands in descending order were albumin, α-1 antitrypsin, plasminogen, fibrinogen, transferrin, apo A-I and E, beta-2 macroglobulin, pre-albumin, apo A-II and A-III, complement components C3, C4 and C5, C-reactive protein, and apo C-2 and C-3. All markers examined were detected with the exception of α-fetoprotein, which is a marker for poorly differentiated hepatocytes.

Example 2

HCV Particles

A. Virus Particle Isolation

Infected primary hepatocyte cells from above were grown on coverslips and analyzed at various times during the culture period for the presence of a novel HCV virus-associated antigen that can be detected by immunocytochemical staining (Burk). Typical cytoplasmic staining was observed in all samples examined, with a tendency for the percentage of cells expressing this marker to increase with time in culture. However, the number of cells with definitive staining never increased above 10%.

To examine the culture medium for HCV particles, culture media were collected on days 11 and 13 in cultured hepatocytes from a second chimpanzee (PTTx174) which had been infected in vivo by inoculation with acute phase plasma from chimpanzee PTTx7.

A sample of culture medium was clarified by centrifugation at 12,000 x g, 30 min. at 4°C. The clarified medium (23 ml) was layered over a discontinuous sucrose gradient formed of 5 mls of 68% sucrose in phosphate buffered saline (PBS), and 10 ml of 20% sucrose. The layered material was centrifuged at 27,000 rpm (131,000 x g) in a Beckman SW28 rotor for 3.8 hours. A 2 ml sample at the 68%/20% sucrose interface was drawn off and diluted to a final sucrose concentration of 20% with PBS. Several gradients (4-6) were prepared from the media collected from each time point.

The diluted material (12.5 ml) was layered over 1 ml of the 68% sucrose solution and centrifuged at 30,000 rpm (154,000 x g) for 16 hours in a Beckman SW41 rotor. The interface material (at the top of the 68% sucrose layer) was collected by bottom puncture, collecting 1-1.5 ml of material. The isolated material, containing purified HCV virus, was frozen at -85°C.

B. Morphology

Purified HCV virus specimens from above were examined by a modification of the pseudoreplication technique (Portnoy). Briefly, 10 µl of virus-containing fluid was pipetted onto agar disks (2% in 0.15M NaCl, 0.01% merthiolate). The agar disk surfaces were covered with a Parlodion film, 0.75% in amyl acetate (Mallinckrodt, Paris, KY). The film containing the HCV viral agent was floated onto a

liquid surface in a 1% phosphotungstic acid, pH 7.0, and retrieved by immersion onto copper grids (3mm). After drying, the specimens were examined by transmission electron microscopy without further treatment.

Virus particles were observed in the samples obtained from the purified tissue culture medium. Figures 1-3 are electron micrographs of the observed material. The morphological features of the particles are described above.

Example 3

HCV Particles from Cultured Cells are Infectious

The production of infectious virus in the hepatocyte cultures was assayed by inoculation of a chimpanzee with tissue culture medium and monitoring the animal for signs of disease. Tissue culture medium as described above was collected from PTTx7 hepatocyte cultures at two-day intervals, and passed through 0.45 μ filters and stored at -100° C. A pool of media from days 3 through 31 were collected (190 ml total) and concentrated by pressure dialysis under N_2 gas at 4°C with an exclusion membrane of 30,000 MW (YM30, Amicon, Beverly, MA)). An 8-fold concentrate (22 ml) was stored at -100°C until use. The concentrated material (10 ml) was used to inoculate an HBV-immune chimpanzee (PTTx196).

Weekly blood samples and periodic liver needle biopsies were obtained from PTTx196 for analysis. A slight increase in ALT occurred during week 4 and microscopic examination of a liver needle biopsy at that time revealed minimal changes similar to those observed in normal tissue, but of interest under these conditions. Liver needle biopsies taken during weeks 8 and 12 exhibited essentially

normal tissue with no microscopic lesions recognized. Due to the delay in onset of clinical hepatitis, a second injection of the same inoculum (7ml) was administered at week 12. This was followed by an elevation in ALT values three weeks later. Peak ALT was exhibited 16-20 weeks after the first inoculation. Histologic examination of a liver needle biopsy taken at week 14 showed early signs of hepatitis, including foci of inflammatory cells in the hepatic parenchyma, and hydropic generation of hepatocytes with occasional necrotic hepatocytes. Electron microscopic examination of the biopsy revealed the presence of cytoplasmic tubules which are typical of HCV-infected tissue.

Plasma samples taken from PTTx196 during weeks 0, 18 and 23 of this experimental HCV infection were analyzed for an increase in antibody titer to cytomegalovirus, Epstein-Barr virus, herpes simplex virus, HBV surface and core antigens (HBsAG, HBcAG), and spumavirus, since these agents may cause hepatitis or could be transmitted by this methodology. No change in the antibody response to these agents was detected in the plasma samples from PTTx196. These results demonstrate that the disease transmitted to PTTx196 was caused by an HCV agent.

Example 4

Genomic Sequence of the HCV Particles

Chimpanzee PTTx198, an 8 year old male chimpanzee, had been inoculated with the Hutchinson strain of HCV virus used in Example 2. During the acute phase of the infection, a liver wedge was used to isolate hepatocytes, which were cultured as HCV-infected primary hepatocytes as describ d in Example 1. The culture medium was used to purify virus as detailed in Example 2. HCV particles, of

the type seen in Figures 1-3 and reported in Example 2, were observed.

A second portion of the biopsied liver from the HCV-infected animal was used to isolate total RNA by the conventional guanidinium isothiocyanate extraction and ultracentrifugation through a cesium chloride gradient (Sambrook). The RNA was used for cDNA synthesis with a specific hepatitis C virus (HVC) oligodeoxyribonucleotide as a primer for reverse transcription. The primer for cDNA synthesis was derived from a previously reported primer (Kubo), and has the sequence:

(5′-GGAAGCTTGACATGCATGTCATGATGTA-3′)

The primer includes 20 nucleotides of HCV specific sequence and 8 nucleotides at its 5′ end containing a HindIII restriction site for subsequent cloning purposes. The reverse transcription was performed as described (Sambrook) in the presence of 5 μg of RNA, 0.5 ug of 3′ primer, 2 units of reverse transcriptase (E. Anglian Biotech, Cambridge, MA) in a 10 μl reaction volume containing 50 mM Tris-HCL pH 8.2, 6mM MgCl2, 10 mM dithiothreitol (DTT) and 500 μM of each of the four deoxyribonucleotide triphosphates (dNTP).

After incubation for 40 minutes at 42°C, 1 μl of the reaction mixture was added to a PCR reaction mixture provided in a commercial PCR kit (Perkin-Elmer/Cetus), as described by the manufacturer. The above 3′-end primer and a 5′-end primer having the sequences

(5′-GGGAATTCGGCTATACCGGCGACTTCG-3′)

which includes 20 nucleotides of HVC sequence (Kubo) and an additional 8 nucleotides at its 5′ end and an EcoR1 site, were added to the reaction mixture. The PCR reaction was allowed to proceed for 30 cycles.

The 623 basepair cDNA fragment amplified by PCR was visualized on an agarose gel by ethidium staining. This fragment was gel purified and amplified again by 30 cycles of PCR. The resulting DNA was gel purified, digested with EcoR1 and HindIII and cloned into the EcoRI/HindIII site of plasmid pGEMX1 (Promega, Madison, WI). The nucleotide sequence was determined by dideoxy chain termination method on double stranded DNA using the SP6 and T3 promoter primers (Promega).

Example 5

HCV Particles from In Vitro Infected Primary Cells

In Example 2, HCV particles were obtained from primary hepatocytes which were infected in vivo, i.e., prior to culturing as primary hepatocytes. In the present example, uninfected chimpanzee primary hepatocytes in culture were infected with HCV in culture, and the virus was allowed to replicate in the infected cells.

Liver wedge biopsies were obtained from healthy, uninfected chimps identified as PTTx266, a 5 year old male chimpanzee, and PTTx344, a 1 year old female chimpanzee. The liver biopsies were used to produce primary cultured hepatocytes, according to the methods detailed in Example 1. The cells were infected with a pool of acute phase plasma from HCV-infected chimpanzees. The virus stock was a pool of acute-phase plasma from HCV-infected chimpanzees. The stock was diluted five-fold in SFM and added to the cultures. The cultures were incubated for 3 hr at 37°C with the inoculum, and then 1.5 ml of SFM was added to the cultures and the incubation was continued for 16 hr. The cultures were washed three times with WME to remove residual inoculum and changed to SFM.

A sample of culture medium was taken at days 1, 2, 3, 6, 9, 12, 15, 18, and 21 for the PTTx266 animal, and at days 1, 3, 5, 7, 9, and 12 for the PTTx344 animal. Virus particles were isolated from culture medium by sucrose gradient centrifugation, as detailed in Example 2. The samples were examined by electron microscopy to determine viral counts. The results are shown in Table 1 above, expressed as virus particles per ml of culture medium.

The table also shows virus counts observed for culture medium obtained at various times after initial culturing, for cell cultures derived from liver cells of HCV-infected chimpanzees, PTTx196, PTTx174, PTTx268, and PTTx198.

Example 6

Immortalized Uninfected Hepatocytes

Uninfected primary chimpanzee hepatocytes derived from PTTx266 were cultured in SFM, substantially as described in Example 1 for HCV-infected cells. The cells were immortalized with a retrovirus derived from the U19-5 cell line which constitutively produces the U19 amphoteric retrovirus. The U19-5 cell line was a gift from Drs. P.S. Jat and P.A. Sharp, M.I.T. (Cambridge, MA). The retrovirus recombinant plasmid construct has been described in detail (Jat). The plasmid construct produces a large T antigen protein defective for binding to the SV40 origin of replication.

The U19-5 cell line was grown in DMEM medium with 10% fetal bovine serum (FBS) under standard culture conditions (Jat). Culture medium was collected at 24-hour intervals and passed through a 0.45 μm filter (Amicon, Beverly, CA)) prior to use for infection of primary hepatocyte cultures.

Subconfluent cultures of primary hepatocytes (Example 1) were infected one day post-plating by the addition of 1 ml of U19-5 culture medium to the cells in the presence of Polybrene™ (8 μg/ml). The plating density was such as to allow the cells several rounds of cell division to occur after introduction of the oncogene. After incubation overnight, cells were washed three times with WME and maintained in SFM until colony outgrowths were observed, typically about 1 month after infection.

The cells were selected for G418 (Geneticin, GIBCO, Gaithersburg, MA) resistance by addition to the culture medium of G418 (400 μg/ml). The cells were then treated by a collagenase/dispase (Boehringer Mannheim) solution at a concentration of 100 μg/ml in phosphate-buffered saline (PBS, pH7.2) for 10 minutes at 37°C. Following dissociation, a five-fold excess of 5% fetal bovine serum in Williams medium E (5% FBS/WME) was added to the solution. Cells were pelleted at 50 x g for six minutes, resuspended in a minimal volume of 5% FBS/WME and allowed to attach during a 2-3 hour period at 37°C under 10% CO_2. The cells were plated at a low cell density so that single colony outgrowths could be isolated and subcloned. From over 100 colonies, over 70 were picked based upon differences in morphological appearance. The cell lines are designated CHMP cells, and are assigned cell line numbers, such as CHMP 1.21, CHMP 1.22, etc.

Example 7

HCV Infectivity of Immortalized Hepatocytes

Immortalized chimpanzee hepatocytes derived from HCV-infected primary hepatocytes were prepared substantially as described in Example 6, but using hepatocytes obtained from

a liver biopsy of a chimpanzee (PTTx198) with acute-phase HCV. The cell lines are designated CU cell lines.

Several CHMP (Example 6) and CU cell lines were cultivated on collagen coated 25 cm^2 Primaria flasks in SFM under normal conditions (37°C, 10% CO_2 atmosphere). When the cultures reached a level of 90% confluency, they were inoculated with chimpanzee plasma known to contain HCV. The inoculum was a pool of plasmas obtained from three chimpanzees (PTTx7, PTTx268, and PTTx174) during the acute phase of a HCV infection and did not contain any other infectious agent. The plasmas were diluted 5-fold in SFM and 1 ml was added to the cultures. After incubation for 3 hr at 37°C, another 3 ml of SFM was added to the cultures and the incubation was continued for 16 hr. The cultures were washed three times with WME to remove the inoculum and SFM was added. The medium was changed every other day and on the 11th day after infection the cultures were harvested for analysis.

B. RNA Characterization

The cells were washed three times with phosphate buffered saline (PBS) and the cellular RNA was extracted and purified using a standard GITC extraction procedure (Chomozynski). The cells were lysed with a solution containing 4M guanidine isothiocyanate, 0.18% 2- mercaptoethanol, and 0.5% sarcosyl. The cell lysate was extracted several times with acidic phenol-chloroform- isoamyl alcohol, and the RNA was precipitated with isopropanol. The purified RNA was resuspended in water and one tenth of each sample was used for polymerase chain reaction (PCR) amplification to detect the HCV RNA genome.

PCR was conducted using standard methodology, as detailed above (Innis). The first step involved a cDNA reaction in which a DNA copy of the HCV RNA was made using reverse transcriptase and an oligonucleotide primer designated 6A that is complementary to the strain of HCV used in our studies. The four primers used for cDNA and PCR were derived from the putative nonstructural region of HCV designated NS3 and their sequences are given below.

Primers:

5A 5′ TCTGTGATAGACTGCAACACG 3′

6A 5′ TTTGGTGATTGGGTGCGTCAG 3′

5B 5′ GATGCTGTCTCCAGGACTCAA 3′

6B 5′ AACAGCGCCCAGTCTGTATAGCAG 3′

The sequence of these primers was derived from the sequence of a cDNA clone of a strain of HCV as previously described (Jacob, 1991). A portion of the cDNA reaction mixture (1/4th) was PCR amplified for 35 cycles using the Taq polymerase and the oligonucleotide primers 5A and 6A. A portion of the first round of PCR (1/50th) was used for a second round of PCR using the primers 5B and 6B.

Figure 5 shows a gel analysis of the PCR products from HCV-infected CU cell lines. Figure 6 shows a gel analysis of PCR products from HCV-infected CHMP cell lines. The results of the gel studies are described above.

Although the invention has been described with respect to particular cell lines and modes of HCV infection, it will be apparent various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A purified hepatitis C (HCV) virus composition comprising virus particles characterized by:

(a) a single-strand RNA genome;

(b) a flavivirus type structure having virus particle sizes between about 30-60 nm, enveloped capside structures, external stalks 2-5 nm in length and width, and an icosahedron symmetry, and

(c) immunospecific reaction with HCV-infected individuals.

2. The composition of claim 1, wherein the RNA genome contains a region with the sequence shown in Figure 4.

3. The composition of claim 1, in which the RNA genome is inactivated.

4. The composition of claim 3, for use as a vaccine, which further includes a suitable vehicle for injecting the virus in a suspended form.

5. A protein composition containing a mature hepatitis C virus (HCV) protein selected from the group of C, e1 (gp35), e2 (gp70), and the HCV counterparts to NS2, NS3, NS4, and NS5, substantially free of HCV genomic RNA, and non-viral serum and cell proteins normally associated with HCV virus infection in humans.

6. The composition of claim 5, wherein the selected protein is substantially purified from the other HCV proteins in the group.

7. The composition of claim 6, for use in a system for detecting the presence of HCV-specific antibodies in human sera, wherein the protein is bound to a solid support, and the system further includes means for detecting the presence of human antibody bound to the support.

8. The composition of claim 7, wherein the protein is gp35 or gp70.

9. The composition of claim 5, for use as a vaccine, which further includes a suitable vehicle for injecting the protein in a suspended form.

10. The composition of claim 9, wherein the protein is gp35 or gp70.

11. A hepatitis C virus (HCV) antigen containing a glycopeptide epitope contained in hepatitis C virus gp35 or gp70 proteins derived from intact HCV particles.

12. The antigen of claim 11, for use in a system for detecting the presence of HCV-specific antibodies in human sera, wherein the antigen is bound to a solid support, and the system further includes means for detecting the presence of human antibody bound to the support.

13. An antibody immunospecific against a hepatitis C virus (HCV) glycopeptide epitope contained in hepatitis C virus gp35 or gp70 proteins derived from intact HCV particles.

14. The antibody of claim 13, for use for use in detecting HCV infection in human sera.

A

Fig. 1A

B

Fig. 1B

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2D

Fig. 2E

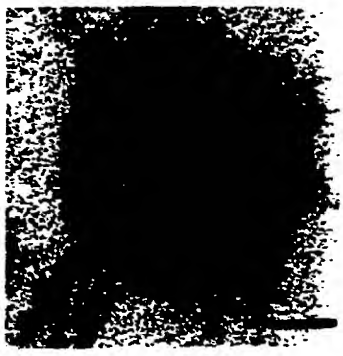

Fig. 2F

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PT                                        G                    T
J1                                        A       C        T
BTR GGC TAT ACC GGC GAC TTC GAC TCT GTG ATA GAC TGC AAC
     Gly Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp Cys Asn

               C                                             C
  A                      G                    T G   T   C   C
ACG TGT GTC ACT CAG ACA GTC GAT TTC AGC CTT GAC CCT ACT
 Thr Cys Val Thr Gln Thr Val Asp Phe Ser Leu Asp Pro Thr

  C                    T
  C       C        G   G   C G G        A       G        G
TTT ACC ATT GAG ACA ACC ACG CTC CCC CAG GAT GCT GTC TCC
Phe Thr Ile Glu Thr Thr Thr Leu Pro Gln Asp Ala Val Ser

C C             T                   T
C C   G   G   G   A   T           T             C  G  AG
AGG ACT CAA CGC CGG GGC AGG ACC GGC AGG GGG AAG CCA
 Arg Thr Gln Arg Arg Gly Arg Thr Gly Arg Gly Lys Pro

            C                                   G
                G           A T   A   A         G        G
GGC ATC TAT AGA TTT GTG GCA CCG GGG GAA CGC CCC TCC
 Gly Ile Tyr Arg Phe Val Ala Pro Gly Glu Arg Pro Ser

                                                  T       CA
 CG           T   T   G       A             T   T       C
GGC ATG TTC GAC TCG TCC GTC CTC TGT GAG TGC TAC GAC GAG
 Gly Met Phe Asp Ser Ser Val Leu Cys Glu Cys Tyr Asp Glu

                                                T       C T G
GGC TGT GCT TGG TAT GAG CTC ACG CCC GCC GAG ACT ACA
 Gly Cys Ala Trp Tyr Glu Leu Thr Pro Ala Glu Thr Thr

         T G   G   T     C A   T   A   A       T G
GTT AGG CTA CGA GCG TAC ATG AAC ACC CCG GGG CTT CCC
 Val Arg Leu Arg Ala Tyr Met Asn Thr Pro Gly Leu Pro
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Fig. 4

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  C                       G   G   C           A           C
GTG TGC CAG GAC CAT CTT GAA TTT TGG GAG GGC GTC TTT
Val Cys Gln Asp His Leu Glu Phe Trp Glu Gly Val Phe

  A
  A             C   C       C           C T G
ACG GGC CTC ACT CAT ATA GAT GCC CAC TTT CTA TCC CAG
Thr Gly Leu Thr His Ile Asp Ala His Phe Leu Ser Gln

                                  C
  T         GCA   A   C       C   C                   A
ACA AAG CAG AGT GGG GAG AAC TTT CCT TAC CTG GTA GCG
Thr Lys Gln Ser Gly Glu Asn Phe Pro Tyr Leu Val Ala

                  A           C         A G   T   A   T
TAC CAA GCC ACC GTG TGC GCT AGG GCT CAA GCC CCT CCC
Tyr Gln Ala Thr Val Cys Ala Arg Ala Gln Ala Pro Pro

                                            T       C
                  T   A                   C C       G   A
CCA TCG TGG GAC CAG ATG TGG AAG TGT TTG ATC CGC CTT
Pro Ser Trp Asp Gln Met Trp Lys Cys Leu Ile Arg Leu

  G
  G   T   G   G   C           G           G   T   G
AAA CCC ACC CTC CAT GGG CCA ACA CCC CTG CTA TAC AGA
Lys Pro Thr Leu His Gly Pro Thr Pro Leu Leu Tyr Arg

                              A
  A   A   C   C           G           C   A       T
CTG GGC GCT GTT CAG AAT GAA GTC ACC CTG ACG CAC CCA
Leu Gly Ala Val Gln Asn Glu Val Thr Leu Thr His Pro

G
  A
ATC ACC AAA TAC ATC ATG ACA TGC ATG TC
Ile Thr Lys Tyr Ile Met Thr Cys Met
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Fig. 4(con't)

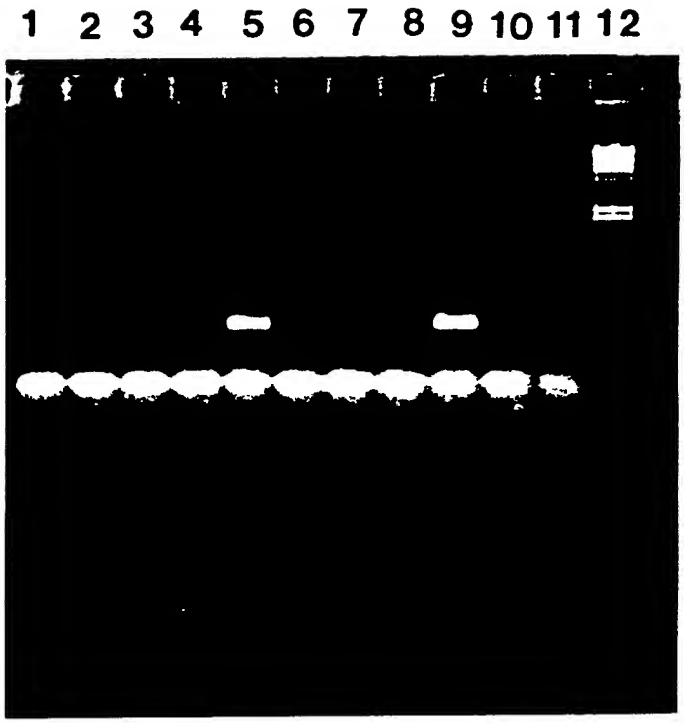

Fig. 5

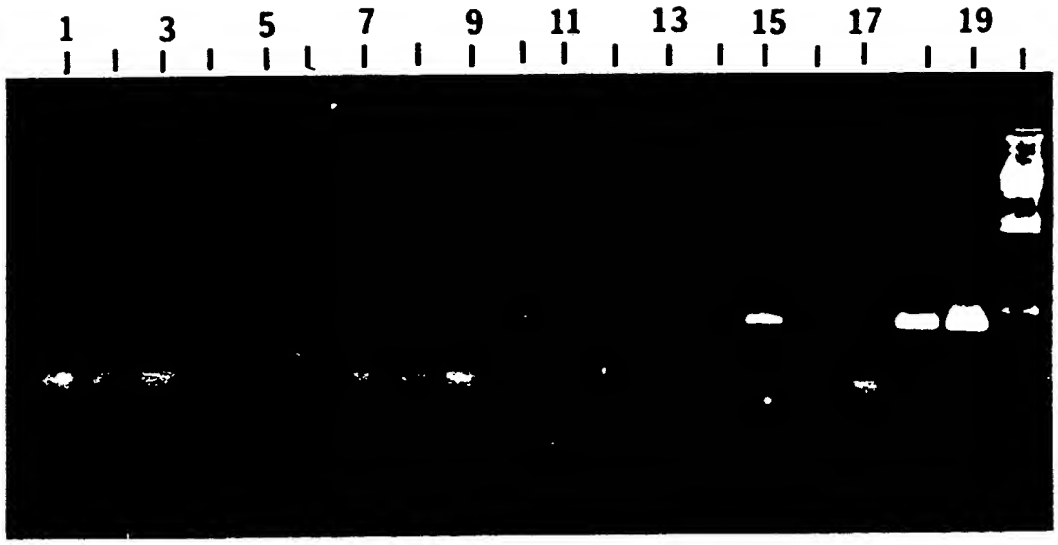

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02298

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 7/00 A 61 K 39/29 C 07 K 15/00
G 01 N 33/576

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 N A 61 K G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO-A-9 000 597 (GENELABS INCORPORATED) 25 January 1990, see the whole document ---	1-14
X	WO-A-8 200 205 (BAXTER TRAVENOL LABORATORIES) 21 January 1982, see the whole document ---	1-4
A	WO-A-8 705 930 (GENELABS INCORPORATED) 8 October 1987 ---	
P,X	WO-A-9 010 060 (SOUTHWEST FOUNDATION FOR MEDICAL RESEARCH) 7 September 1990, see examples 3,4 ---	1-4
P,X	EP-A-0 414 475 (CHIRON CORP.) 27 February 1991, see page 14, lines 17-55; claims 11-17 --- -/-	1-14

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31-07-1991	18. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van d r Haas

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	JOURNAL OF INFECTIOUS DISEASES, vol. 161, June 1990, J.R. JACOB et al.: "Expression of infectious viral particles by primary chimpanzee hepatocytes isolated during the acute phase of non-A, non-B hepatitis", pages 1121-1127, see the whole document -----	1-14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9102298 SA 46586

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/09/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9000597	25-01-90	AU-A- 4046489	05-02-90
		EP-A- 0423239	24-04-91
WO-A- 8200205	21-01-82	US-A- 4464474	07-08-84
		EP-A,B 0058676	01-09-82
WO-A- 8705930	08-10-87	AU-A- 7238387	20-10-87
		EP-A- 0261233	30-03-88
		JP-T- 63502962	02-11-88
WO-A- 9010060	07-09-90	AU-A- 5166690	26-09-90
EP-A- 0414475	27-02-91	AU-A- 6344990	03-04-91
		WO-A- 9102820	07-03-91